Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 291 (2006) 12 – 24

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Distinct roles for retinoic acid receptors alpha and beta in early lung morphogenesis

Tushar J. Desai [a,1], Felicia Chen [a], Jining Lü [a], Jun Qian [a], Karen Niederreither [c,d], Pascal Dollé [b], Pierre Chambon [b], Wellington V. Cardoso [a,*]

[a] *Pulmonary Center, Boston University School of Medicine, 80 East Concord Street R-304, Boston, MA 02118, USA*
[b] *Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP/College de France, BP 10142, 67404 Illkirch Cedex, C. U. De Strasbourg, France*
[c] *Department of Medicine, Center for Cardiovascular Development, 506D, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*
[d] *Department of Molecular and Cellular Biology, Center for Cardiovascular Development, 506D, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

Received for publication 26 May 2005; revised 5 September 2005, accepted 3 October 2005
Available online 19 January 2006

Abstract

Retinoic acid (RA) signaling is required for normal development of multiple organs. However, little is known about how RA influences the initial stages of lung development. Here, we used a combination of genetic, pharmacological and explant culture approaches to address this issue, and to investigate how signaling by different RA receptors (RAR) mediates the RA effects. We analyzed initiation of lung development in retinaldehyde dehydrogenase-2 (*Raldh2*) null mice, a model in which RA signaling is absent from the foregut from its earliest developmental stages. We provide evidence that RA is dispensable for specification of lung cell fate in the endoderm. By using synthetic retinoids to selectively activate RAR alpha or beta signaling in this model, we demonstrate novel and unique functions of these receptors in the early lung. We show that activation of RAR beta, but not alpha, induces expression of the fibroblast growth factor *Fgf10* and bud morphogenesis in the lung field. Similar analysis of wild type foregut shows that endogenous RAR alpha activity is required to maintain overall RA signaling, and to refine the RAR beta effects in the lung field. Our data support the idea that balanced activation of RAR alpha and beta is critical for proper lung bud initiation and endodermal differentiation.

Keywords: Lung development; Retinoic acid; Foregut; *Raldh2*; Fgf10; Organogenesis; Endodermal specification; RAR; SftpC; *Nkx2.1*

Introduction

Retinoic acid (RA), the active form of vitamin A, is essential for vertebrate organogenesis (Wilson et al., 1953). In mice, genetic disruption of retinaldehyde dehydrogenase 2 (*Raldh2*), an enzyme essential for RA synthesis, leads to major defects in cardiovascular and axial development, and early embryonic lethality (Niederreither et al., 1999; Mic et al., 2002).

The murine lungs emerge at around embryonic day E9.5 as two endodermal buds from the ventral–lateral portions of the primitive foregut, and subsequently undergo branching morphogenesis to generate the airway tree (Hogan, 1999; Cardoso, 2001; Shannon and Hyatt, 2004). Previous analysis of the ontogeny of retinoid signaling in the mouse embryo indicates that RA synthesis and utilization are prominent in the foregut at the onset of lung development (Malpel et al., 2000). *Raldh2* is strongly expressed by the mesoderm and mesothelial surfaces of the E8.5–9.5 foregut. Analysis of embryos harboring a RA-responsive (*RARElacZ*) transgene (Rossant et al., 1991) reveals widespread RA-response in all layers, suggestive of ubiquitous activation of RA receptors (RARs). However, by E10.5–E11.5, as secondary buds form, RA signaling is dramatically downregulated in nascent epithelial buds. This downregulation appears to be required for proper development of the distal lung (Wongtrakool et al., 2003). The observations above support the idea that critical RA-dependent events occur during the initial stages of lung development. Indeed, lung agenesis has been

* Corresponding author. Fax: +1 617 536 8093.
E-mail address: wcardoso@lung.bumc.bu.edu (W.V. Cardoso).
[1] Present address: Biochemistry Department, Stanford University, Beckman 463, Stanford, CA 94305, USA.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.10.045

reported in rat embryos under severe vitamin A deficiency (VAD), and in other animal models in which RA signaling has been antagonized (Mollard et al., 2000; Dickman et al., 1997).

In spite of much evidence linking lung defects with altered retinoid signaling, the molecular mechanisms by which endogenous RA influences lung development remain little understood. Using a mouse foregut explant culture system, we have recently shown that, within a limited developmental window (8–15 somite stages), endogenous RA is selectively required in the prospective lung field for proper mesodermal proliferation and induction of *Fgf10* expression (Desai et al., 2004). Fgf10, in turn, is essential to activate Fgfr2 signaling in the endoderm and induce bud morphogenesis (Sekine et al., 1999; De Moerlooze et al., 2000; Min et al., 1998).

Another study using recombinant tissue explant cultures has implicated FGF signals from the cardiac mesoderm as initiators of lung endodermal fate in the 7–8 somite stage foregut (Serls et al., 2005). We have previous evidence that RA signaling is necessary to maintain expression of *Nkx2.1*, a homeobox gene also known as thyroid transcription factor 1 (*Ttf1*), which is an early marker of lung endodermal cells (Desai et al., 2004). Whether RA is required for lung specification in the early foregut endoderm has not been determined. In previous model systems that focused on the lung, this issue could not be properly addressed because the endoderm had been exposed to endogenous RA prior to culture (Mollard et al., 2000). Thus, the role of RA in lung specification remains unresolved.

RA signaling is mediated by two families of nuclear receptors, the RARs and RXRs, each consisting of three isotypes alpha, beta and gamma. Genetic deletion of these receptors results in complex phenotypes that altogether recapitulate the abnormalities described in VAD rodents (Wilson et al., 1953; Kastner et al., 1997; Chambon, 1996; Clagett-Dame and DeLuca, 2002). Analyses of the various RAR double mutants implicate RAR alpha and beta as critical receptors for the developing lung. Abnormalities in RAR alpha and beta double null mice include unilateral lung agenesis and unilateral or bilateral lung hypoplasia (Mendelsohn et al., 1994a). Intriguingly, RAR single knockout mice show no abnormal lung phenotype (Li et al., 1993; Mendelsohn et al., 1994b). Although these studies inarguably implicate these receptors in lung morphogenesis, they do not define a specific role for individual RARs in this process. Thus, the presumed role of each of these receptors in the primordial lung remains largely undemonstrated.

In the present study, we have used pharmacological, genetic and in vitro approaches to study the role of RA in lung specification, and to find how signaling by different RARs mediates the RA effects in the early lung. For this, we analyzed initiation of lung development in wild type and *Raldh2*$^{-/-}$ mice, a genetic model in which RA signaling is absent in the foregut from its earliest stages. By using synthetic retinoids to selectively activate RAR alpha or beta signaling in these mutants or in wild type mice, we demonstrate novel and unique functions of these receptors in the early lung. We provide evidence that RA is dispensable for specification of lung cell fate in the endoderm. We show that in the absence of endogenous RA, activation of RAR beta, but not alpha, induces *Fgf10* and bud morphogenesis. Analysis of the wild type foregut shows that endogenous RAR alpha activity is required to maintain overall RA signaling and refine the RAR beta effects in the developing foregut. Our study suggests that a proper balance of activation of RAR alpha and beta during early lung development is critical for lung bud initiation and endodermal differentiation.

Materials and methods

Raldh2 null mutant mice

The generation of these mutants has been previously reported (Niederreither et al., 1999). Disruption of the *Raldh2* gene resulted in the loss of RA signaling activity in all embryonic structures except the developing eye. E8.5-null mutant mice were distinguished from their heterozygous and wild type littermates by distinct phenotypical features, such as large unseptated heart that did not undergo looping, abnormally small somites, and no axial rotation. For experiments involving foregut explant cultures, *Raldh2*-null mutants were genotyped by polymerase chain reaction (PCR) analysis of embryonic tissues, as previously described (Niederreither et al., 1999).

RARElacZ reporter mice

This mouse line has been previously described (Rossant et al., 1991). These mice carry the bacterial *lacZ* gene under the control of a heat shock minimal promoter (hsp68) and three copies of the RA-response element (*RARE*) from the *RAR-beta2* promoter (Rossant et al., 1991). *LacZ* is thus expressed at sites where endogenous RA or exogenous retinoids activate RARs. Beta-galactosidase staining was performed overnight using X-gal as substrate, as described (Malpel et al., 2000). Subsequently, some explants were processed for histological analysis of 5 μm paraffin sections stained with Hematoxylin and Eosin (H&E).

Foregut explant cultures

Timed pregnant wild type CD1 (Charles River), *RARElacZ*, or *Raldh2* mutant mice were sacrificed at gestational day 8.5 (E8.5). Experiments were performed with foreguts dissected from 8- to 15-somite-stage embryos as previously described (Desai et al., 2004). Explants were cultured for 1–6 days on Costar 6-well Transwell-Col plates containing 1.5 ml of BGJb medium (GIBCO-BRL), 10% fetal calf serum (GIBCO-BRL), penicillin–streptomycin, and 20 mg/dl ascorbic acid (Sigma) with or without selective retinoid agents (see below) or *all-trans* RA (Sigma). Cultures were shielded from light and incubated at 37°C in 95% air and 5% carbon dioxide. Medium was changed daily. In some experiments, heparin beads soaked in human recombinant FGF10 (100 ng/μl, R&D Systems) or PBS were grafted onto foregut explants. Explants were processed for X-gal staining (*RARElacZ* mice), snap-frozen or fixed in 4% paraformaldehyde for whole mount in situ hybridization (ISH).

Modulation of RA signaling

We used *all-trans* RA (Sigma), as well as the selective RAR-alpha antagonist BMS-189532, RAR-alpha agonist BMS-194753, or RAR-beta agonist BMS-213309 (Bristol Myers Squibb). The selectivity and biological effects of these retinoids have been previously characterized in several reports (Gehin et al., 1999; Matt et al., 2003; Dilworth et al., 1999; Schoenermark et al., 1999; Vivat-Hannah et al., 2001; Chapellier et al., 2002). Compounds were reconstituted in ethanol and stored at −70°C in light-protected conditions. These were then added to BGJb medium either singly or in combination at a final concentration of 10^{-6} M with the exception of the RAR-beta agonist, which was used at 10^{-7} M. For all experiments, conclusions (effects on morphology, gene or protein expression) were based on the evaluation of at least 3 specimens per condition.

In situ hybridization (ISH)

Whole mount ISH of embryos and foregut explants were performed as described (Malpel et al., 2000). Briefly, digoxigenin (DIG)-labeled riboprobes (Maxiscript kit, Ambion) were generated and amplified from plasmids carrying cDNAs for *Nkx2.1* (Parviz Minoo, University of Southern California), *Fgf10* (Nobuyuki Itoh, Kyoto University), *Shh* (Andrew P. McMahon, Harvard Biolabs), *Pdx1* (Chris Wright, Vanderbilt University), and *Sftp-C* (Cardoso et al., 1997). Specimens were rehydrated, digested with Proteinase K, pre-hybridized for 1 h at 70°C in solution containing 50% formamide, 5× SSC buffer, 1% sodium dodecyl sulfate (SDS), 50 mg/ml yeast RNA and heparin, followed by overnight hybridization with DIG-labeled RNA probes. Samples were then incubated with anti-DIG alkaline phosphatase conjugate overnight at 4°C. Signal was visualized with BM Purple substrate solution (Roche Diagnostics), according to the manufacturer's protocol. In addition, non-isotopic ISH was performed in cryosections using RAR cDNA templates for riboprobes as described (Martin et al., 2005).

Cell proliferation assay

Cell proliferation was evaluated by PCNA staining of 5 μm paraffin sections using the Cell Proliferation Kit (Zymed) and the DAB staining kit (Vector Laboratories), according to the manufacturer's recommendations.

RT-PCR

RNAs were isolated from the foregut explants using the RNeasy kit (Qiagen), and reverse transcribed using random primers and SuperScript II reverse transcriptase (Invitrogen). cDNAs from the reverse transcription were used as templates for 40 cycles of PCR, using *SftpC*-specific primers: 5′-CTCGTTGTCGTGGTGATTGT-3′ (forward primer) and 5′-AGCT-TCCTGCTTGTCTGACA-3′ (reverse primer), and beta actin (Malpel et al., 2000).

Quantitative analysis of Fgf10 staining

Following whole mount ISH, explants were post-fixed in 2% glutaraldehyde for 1 h at room temperature, and cleared in a 70% glycerol solution. Specimens were photographed using SPOT imaging software. For quantitative analysis, the average pixel intensity of these images was estimated using IMAGE J software; values were multiplied to the surface area to arrive at an absolute pixel score for each explant. Results were represented in a graphic form (bars) as an average of 3 specimens for each group.

Results

Arrested lung development in Raldh2$^{-/-}$ mice in vivo

Raldh2$^{-/-}$ embryos were promptly recognized by the gross structural abnormalities of the developing heart and pharyngeal region described in previous reports. At E8.5–E10, the foreguts of mutants appeared shorter in length compared to wild type littermates due to overall body truncation and hypoplasia of the posterior branchial arches (Niederreither et al., 2000, 2001; Chazaud et al., 2003).

Histological analysis of E9.0–9.5 mutants indicated that bud morphogenesis occurred at the site of the thyroid, liver and ventral pancreas (Fig. 1A, and data not shown). PCNA staining strongly labeled the endoderm where each of these organ primordia was forming (Fig. 1B left panel). No buds or PCNA activity, however, could be identified in the prospective lung field (right panel in Fig. 1B). The molecular identity of the *Raldh2*$^{-/-}$ foregut derivatives was investigated by whole mount in situ hybridization (WMISH). In the E9–9.5 wild type embryo *Nkx2.1* labels the developing forebrain, thyroid and lung fields (Kimura et al., 1999; Minoo et al., 1999). *Nkx2.1* analysis of E9–9.5 *Raldh2*$^{-/-}$ embryos (15–26 somites) showed preserved expression in the forebrain and thyroid but only inconsistent, near background signals in the endoderm of the prospective lung region (Fig. 1C). WMISH of *Fgf10* showed lack of signals in the lung field of mutants, although expression could be detected elsewhere in the foregut mesoderm (Fig. 1D). The local loss of endodermal proliferation depicted in Fig. 1B (*) was expected, since expression of Fgf10, a major mitogen for airway development in the lung, was lost. The absence of lung buds was consistent with the phenotype reported in other models of severe disruption of retinoid signaling (Dickman et al., 1997; Mollard et al., 2000), and confirmed our previous observation of RA requirement for *Fgf10* induction in the early lung (Desai et al., 2004). Moreover, assessment of the duodenal and pancreatic marker gene *Pdx1* confirmed the agenesis of the dorsal pancreas recently reported in *Raldh2*$^{-/-}$ embryos (Martin et al., 2005; Molotkov et al., 2005).

Altered RAR expression in Raldh2$^{-/-}$ mice

Previous studies have shown that expression of *RAR alpha* and *beta*, critical for early lung development, is initially found in all layers of the foregut; *RAR beta* signals, however, decline in the mesoderm as primary lung buds form (Ruberte et al., 1991). ISH of *RAR alpha* and *beta* demonstrated the pattern reported in E8.5–9.0 wild type foregut. This is shown in the left panels of Figs. 1F, H (whole mounts, top) and Figs. 1G, I (in sections, bottom). In *Raldh2*$^{-/-}$ mutants *RAR alpha* signals were markedly reduced at all levels of the foregut (*, right panel Figs. 1F, G) and midgut (inset in Fig. 1G). *RAR bet*a expression was similarly downregulated in the mesoderm of mutants, but appears to be preserved throughout the gut endoderm (Fig. 1I). The overall downregulation of these receptors in the trunk region of *Raldh2*$^{-/-}$ mutants confirmed their dependence on endogenous RA. In spite of the low levels, these receptors were still able to mediate RA responses, when ligand was provided.

Extended culture of Raldh2$^{-/-}$ explants confirms failure of lung development, which can be rescued by exogenous RA

The severe abnormalities of the *Raldh2*$^{-/-}$ mutants preclude an in vivo analysis of these embryos beyond E9.5–10.5 (Niederreither et al., 2000, 2001; Chazaud et al., 2003). Thus, we could not unambiguously determine if the lack of *Fgf10* signals and lung bud formation reflected an overall developmental delay of the mutant embryos. We have previously characterized an explant culture system in which foregut organogenesis can be followed in vitro under controlled conditions for several days (Fig. 2A) (Desai et al., 2004). We used this system to address this issue. E8.5 *Raldh2*$^{-/-}$ foreguts cultured in control (RA-deficient) medium for up to 6 days were viable and continued to grow, as indicated

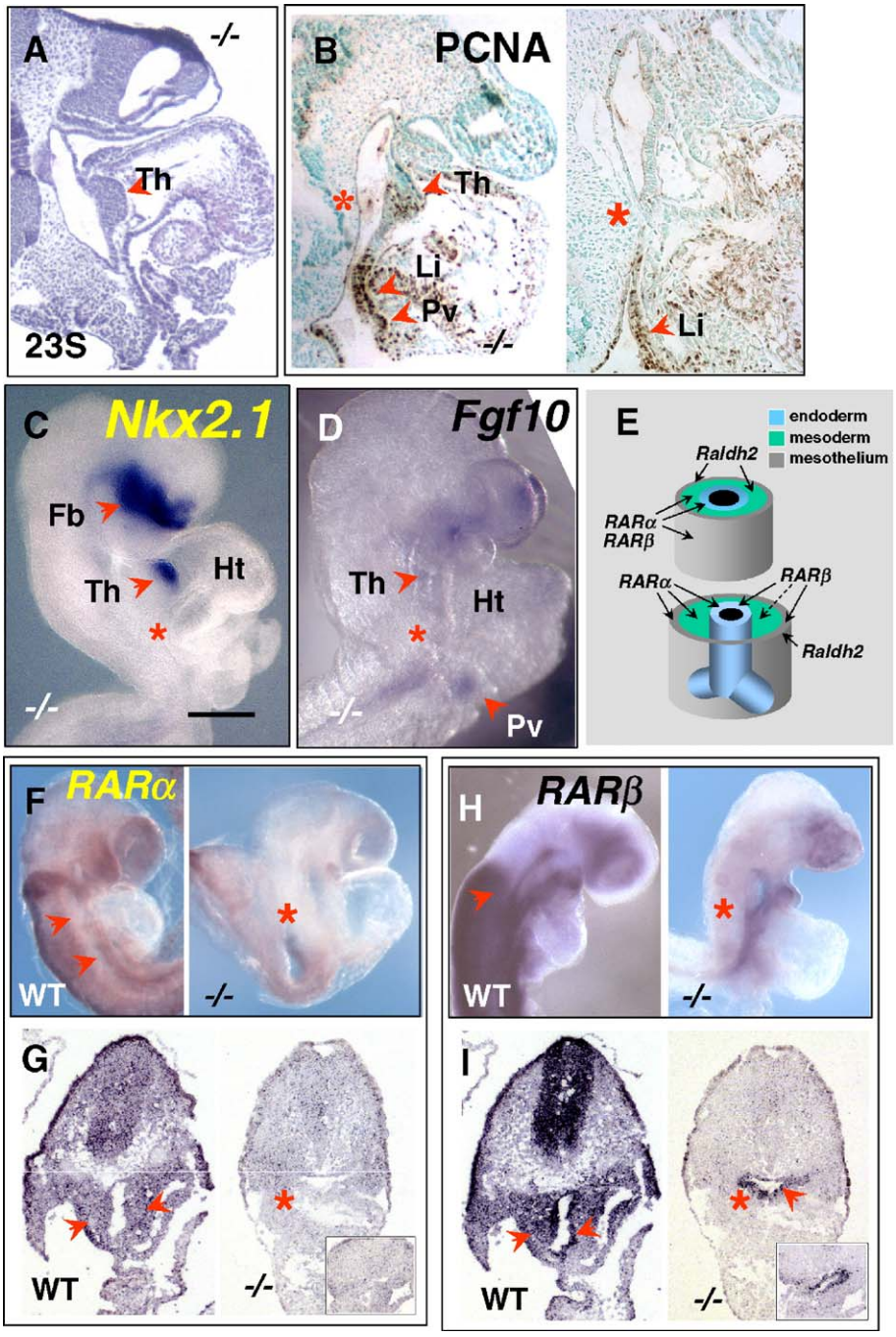

Fig. 1. Disruption of lung development in *Raldh2*$^{-/-}$ mice in vivo. (A) Hematoxylin and eosin staining of 23 somite-stage embryo (E9.5) showing thyroid primordium (Th). (B) PCNA staining (arrowheads) labels the foregut endoderm at the sites of thyroid (Th), liver (Li), dorsal and ventral pancreatic buds (Pv, respectively), but not at the prospective lung region (*, also in right panel). (C) Whole mount in situ hybridization (WMISH) of *Nkx2.1* in 23S mutants shows preserved expression in the forebrain (Fb) and the thyroid, but not in the prospective lung region (*). (D) WMISH for *Fgf10* shows signals in ventral pancreatic primordium (Pv), but not in the prospective lung (*). (E) Diagram representing expression of *Raldh2, RAR alpha,* and *beta* in the wild type foregut before (E8.5, top), and during (E9.5, bottom) primary lung bud formation. *Raldh2* is present in mesothelium and mesoderm at both stages, but is absent from the endoderm. Both *RAR*s are present initially in all layers, but *RAR beta* expression decreases in the mesoderm (dashed arrow) after primary budding. (F) ISH (whole mount and sections) of *RAR alpha* (F, G) and *beta* (H, I) in E8.5–9.0 (15–18S) wild type (WT, left panels) and *Raldh2*$^{-/-}$ (right panels) show marked downregulation of *RAR* expression in mutants (*) as compared to WT (arrowheads). In mutants, *RAR beta* appears more preserved in the endoderm (arrow in I) than in the mesoderm. Insets in panels G, I, depict *RAR* expression in the mutant at a more posterior level (midgut). Scale bar in E, 350 μm. (Ht): heart.

by beating heart tissue and overall increase in size of the explant with time. Foregut morphogenesis, however, was abnormal. There was evidence of bud formation in the thyroid but not in the lung field, even after 6 days of culture (Fig. 2B). This phenotype was reminiscent of that previously described in pan-RAR antagonist-treated foregut cultures, although in most cases, the foregut tube was more distorted and shortened (Desai et al., 2004).

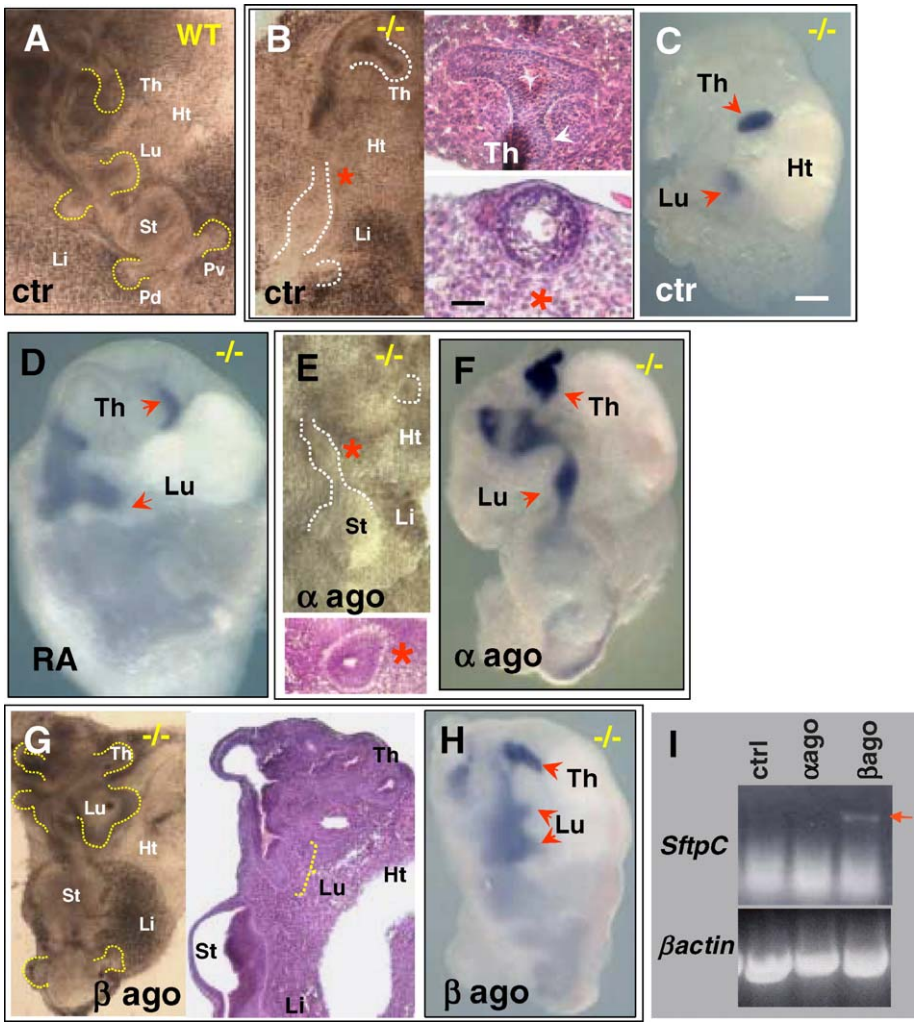

Fig. 2. Foregut organogenesis in explant cultures of *Raldh2*$^{-/-}$ embryos: Effect of RAR agonists. (B, E, G), whole mount specimens (side panels, H&E staining); whole mount in situ hybridization (WMISH) of *Nkx2.1* in *Raldh2*−/− foregut cultures at day 4 in control (C), *all trans* RA 10^{-7} M (D), RAR alpha agonist 10^{-6} M (F), or beta agonist 10^{-7} M (H) supplemented media. (A) Day 4 control wild type foregut cultures; thyroid (Th) and liver (Li) morphogenesis already initiated prior to culture, whereas lung (Lu), stomach (St), and pancreas (Pv, ventral; Pd, dorsal) initiated during culture period. (B) Day 4 *Raldh2*$^{-/-}$ foregut cultures in control medium show no evidence of bud formation in the lung field (*) (right panels: H&E staining of transverse section at the level of the thyroid and unbudded presumptive lung). (C–D) *Raldh2*$^{-/-}$ control cultures do not develop lung buds; however, the presence of *Nkx2.1* signals in the lung and thyroid fields suggests that the lung endoderm has been specified. *all-trans*-RA (D) or RAR beta agonist (G, H), but not alpha agonist (E, F), rescues lung bud formation and show strong *Nkx2.1* staining (H). PCR analysis of *Raldh2*$^{-/-}$ foregut cultures at day 6 shows that the lung marker *SftpC* is induced only by beta agonist. Scale bar in panels B and C, 60 and 300 μm, respectively.

At day 4, *Raldh2*$^{-/-}$ foregut cultures continued to express strong *Nkx2.1* signals in the thyroid primordium; importantly, we could consistently identify *Nkx2.1* labeling in the prospective lung field (Fig. 2C). In spite of this, the presumptive lung endoderm did not further progress into the differentiation program to express the surfactant-associated protein gene *SftpC*.; signals could not be detected either by in situ hybridization or by PCR even at day 6 (Fig. 2I). Culturing *Raldh2*$^{-/-}$ explants in RA-containing medium (10^{-7} M) rescued primary lung budding and local expression of *Nkx2.1* (Fig. 2D). Together, the data suggested that RA signaling is not required to specify lung cell fate in the developing foregut. RA, however, is necessary to maintain a program of differentiation of lung progenitor cells, and to initiate bud morphogenesis in the lung field.

Signaling by RAR beta– but not alpha– rescues lung bud formation in Raldh2$^{-/-}$ *mutants*

To dissect the function of individual RARs during the initial stages of lung development, we differentially activated RA signaling using selective RAR agonists in *Raldh2*$^{-/-}$ foregut cultures. We focused on RAR alpha and beta because of their essential role in the early lung (Mendelsohn et al., 1994a).

We found that treatment with the RAR alpha agonist (BMS 753) at concentrations that elicit widespread *RARElacZ* expression in foregut explants of reporter mice (10^{-7}–10^{-6} M, shown below in Fig. 5B), did not rescue lung bud formation in *Raldh2*$^{-/-}$ explants (Fig. 2E). This was also evident in explants probed for *Nkx2.1*, which in the lung field labels the endoderm that failed to bud (Fig. 2F). No expression of the distal marker gene *SftpC* could be detected even by PCR

analysis (Fig. 2I). In the early lung, proximal and distal respiratory progenitors express *Nkx2.1*, but only the distal lineage is positive for *SftpC* (Perl et al., 2002; Wert et al., 1993; Minoo et al., 1999). Presumably, the *Nkx2.1* positive-*SftpC* negative endodermal cells in RAR alpha agonist-treated cultures represent proximal respiratory progenitors. This raises the possibility that during development proximal and distal precursors have different requirements for RAR signaling.

By contrast, cultures of mutants treated with RAR beta agonist (10^{-7} M) showed *Nkx2.1*-labeled buds at the region of the prospective lung (Figs. 2G, H). PCR analysis revealed *SftpC* expression, a definitive proof of an ongoing program of distal lung differentiation (Wert et al., 1993). These lungs, however, were morphologically different from those seen in *all-trans* RA-rescued explants, as they often appeared as dilated or lobulated structures (Figs. 2G and 3D, high magnification). This suggested that, although morphogenesis could be induced by the beta agonist, additional RAR-mediated signals were required for normal morphology of the lung buds in the *Raldh2*$^{-/-}$ foreguts.

Fgf10 is differentially regulated by RAR beta or alpha agonists

Analysis of 4-day *Raldh2*$^{-/-}$ explant cultures showed that in RA deficient control and RAR alpha agonist-treated foreguts, *Fgf10* signals were absent, or low and diffuse in the mesoderm near the unbudded presumptive lung endoderm (Figs. 3A, B). This differed from the RAR beta agonist-treated cultures, in which strong *Fgf10* expression could be detected throughout the explant, including the region associated with the lung buds (Figs. 3C–E, also in inset in D). The differential effect of beta agonist in inducing bud morphogenesis in the lung field is a function of the ability of RAR beta signaling to induce expression of *Fgf10* in the corresponding mesoderm. As

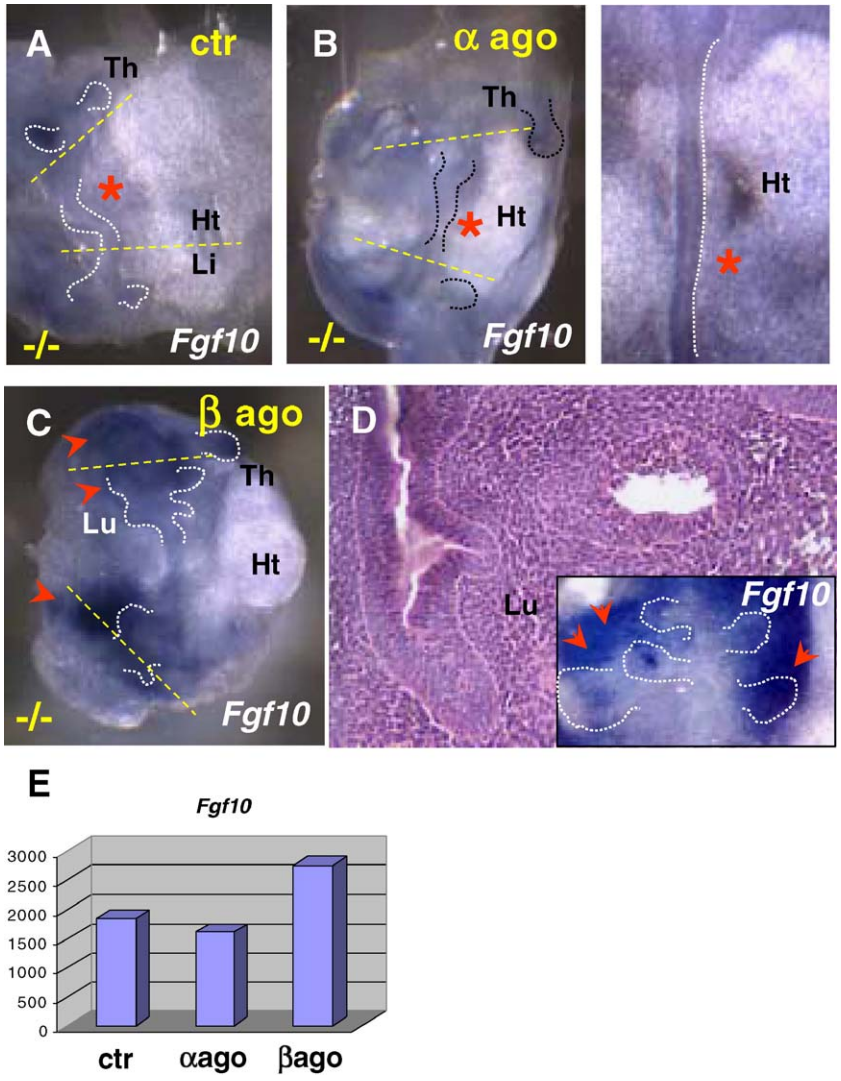

Fig. 3. (A–C) Differential effects of RAR alpha and beta agonists on *Fgf10* expression (WMISH) in day 4 *Raldh2*$^{-/-}$ foregut cultures. In control (A) or alpha agonist-treated cultures (B) *Fgf10* signals are nearly undetectable (*) in the mesoderm of the prospective lung (demarcated by lines), although present elsewhere. (C–D) By contrast, *Fgf10* signals are strong and more broadly distributed in beta agonist-treated explants (arrows in C, also in inset in D); buds do form in the lung field, sometimes multiple and more anteriorly located (D, H&E staining). (E) Quantitative analysis of *Fgf10* staining in these explants (optical density, arbitrary units) confirms increased signals with beta agonist.

suggested by our previous study (Desai et al., 2004), the RA regulation of *Fgf10* may be indirect. Disruption of RA signaling in the foregut selectively disrupts *Fgf10* in the lung field but not at other *Fgf10*-expressing sites, such as the pancreas or thyroid fields. Moreover, after the 15-somite stage, *Fgf10* expression in the lung field is no longer dependent on RA (Desai et al., 2004). Analysis of 10 kb upstream regions of the *Fgf10* promoter reveals no obvious RA responsive element (personal communication: W.V. Cardoso, J. Lu, J. Jean, M. Joyce-Brady). However, we cannot rule out the possibility that a *RARE* may be present in an intronic region or elsewhere.

The augmented and more diffuse expression of *Fgf10* in RAR beta-agonist-treated explants is expected to result in activation of its receptor Fgfr2b in a broader domain. This could account for the abnormally shaped appearance of the lung buds. Together, the data support the idea that RAR alpha and beta have distinct targets and roles in the developing lung.

Raldh2$^{-/-}$ lung endodermal precursors undergo differentiation in response to exogenous FGF10

Our data in *Raldh2$^{-/-}$* foreguts suggested that the lung endoderm was specified. However, further differentiation of distal precursors into *SftpC*-expressing cells occurred only when a retinoid agonist induced *Fgf10* expression in the lung field. Therefore, we asked whether Fgf10 was the critical signal for differential induction of *SftpC* by the RAR beta agonist in *Raldh2$^{-/-}$* explants.

For this, we engrafted onto E8.5 *Raldh2$^{-/-}$* foregut explants heparin beads soaked in human recombinant FGF10 or phosphate-buffered saline (PBS). Then, explants were cultured in control RA-devoid medium. As predicted, FGF10, but not the PBS bead elicited a local budding response in the mutant foregut endoderm (Figs. 4A, B) (Desai et al., 2004). In situ hybridization showed strong *Nkx2.1* signals in the endoderm that grew toward the FGF10 bead. Remarkably, PCR analysis of these explants revealed *SftpC* expression in the endoderm associated with the FGF10, but not the PBS bead (Fig. 4C). These results indicated that in the absence of endogenous RA, exogenous FGF10 is able to maintain and expand a population of *Nkx2.1*-expressing lung endodermal cell progenitors and allow them to further express *SftpC*. Thus, in our model FGF10 substitutes for RAR beta agonist in fostering a program of lung epithelial cell differentiation that initiated at an earlier stage. FGF10 may do so by locally activating Fgf signaling in the foregut endoderm, and by expanding a population of progenitor cells which, then, undergoes a distal differentiation program. An Fgf10 role in maintaining a pool of organ progenitor cells has been demonstrated in other developing structures, such as the tooth buds (Harada et al., 2002; Bhushan et al., 2001).

Balanced activation of RAR isotypes during early lung development: the role of RAR alpha

Although our model suggested a critical role for RAR beta in lung bud initiation, in vivo data from RAR double

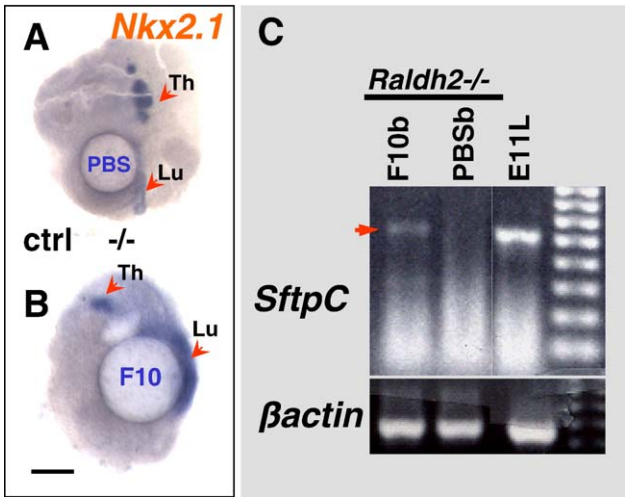

Fig. 4. Exogenous FGF10 in the absence of RA signaling is able to maintain lung endodermal cell progenitors expressing *Nkx2.1* and allows expression of *SftpC*. (A, B) Engraftment of a heparin bead soaked in recombinant FGF10 (F10b), but not buffer (PBS), upregulates and expands *Nkx2.1* expression domain in the prospective lung field of *Raldh2$^{-/-}$* cultures. (C) Semi-quantitative PCR analysis reveals *SftpC* expression (*beta actin* on bottom gel) in cultures engrafted with the FGF10, but not the PBS beads. E11L, embryonic lung positive control. Scale bar in B, 200 μm.

null mutants show that RAR beta disruption is deleterious to the lung only in the context of RAR alpha deficiency (Mendelsohn et al., 1994a). We investigated the possibility that proper RA signaling results from balanced activation of different RAR isotypes at the onset of lung development. For this, we altered the endogenous balance of RAR alpha and beta activation using synthetic retinoids in RA-sufficient *RARElacZ* reporter mice. X-gal staining allowed monitoring RAR activity under the different experimental conditions. To better visualize the effect of the various treatments in foregut morphogenesis, we performed H&E staining in sections of these explants, and we also assessed expression of sonic hedgehog (*Shh*) in whole mounts. Shh labeling facilitated the analysis because this gene is strongly expressed in the foregut endoderm, and signals are not affected by disruption of RA signaling (Desai et al., 2004).

During the initial 18–24 h in culture, control foreguts expressed *RARElacZ* in both endoderm and mesoderm (Fig. 5A). Signals were absent from the most anterior domains, which includes the thyroid primordia. Treatment with the alpha agonist (10^{-6} M) resulted in increased transgene activation throughout the explant, a response consistent with the ubiquitous distribution of RAR alpha (Dolle et al., 1990) (Fig. 5B). By 72 h the *RARElacZ* response was predominantly seen in the foregut endoderm in both controls and alpha agonist explants, although alpha agonist continued to activate *RARElacZ* in the cardiac tissue (Figs. 5E, F, I, J). Interestingly, excess RAR alpha activity had no effect in lung bud morphogenesis as depicted by H&E and *Shh* labeling of the endoderm (Figs. 5 I, J and 6E, F). RAR alpha agonist also had no effect on *Nkx2.1* expression in the lung (Figs. 6A, B).

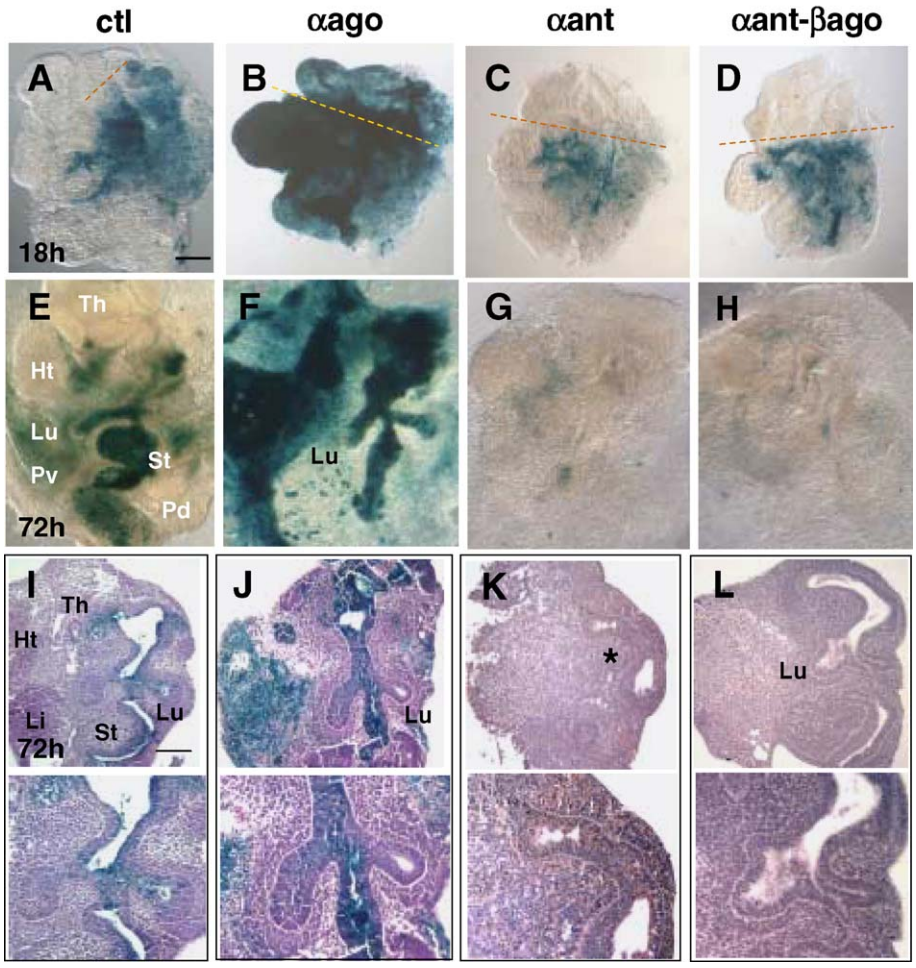

Fig. 5. X-gal staining of *RARElac*Z foregut explants cultured for 18 h (A–D) and 72 h (E–L) with synthetic retinoids. The yellow line depicts the anterior limit of RA response in controls; (B) At 18 h RAR alpha stimulation activates RA signaling ubiquitously; (C) the RA response observed with alpha antagonist likely represents sites of endogenous RAR beta signaling, since when combined with a beta agonist (D), the intensity, but not the distribution of *lac*Z signals changes. (E–H) By 72 h, *RARElac*Z expression is still strong in control and alpha agonist-treated cultures, but is no longer seen in explants treated with alpha antagonist (G), even if combined with beta agonist (H). (I–L) H&E stained histological sections of 72 h *RARElac*Z cultures (lower panel: high magnification of the lung field). (J) Alpha agonist has minimal or no effect in the explant, however (K) alpha antagonist disrupts foregut morphogenesis; lung buds (*) appear truncated or not formed and the foregut tube morphology is altered; (L) beta agonist partially restores the organization of the foregut. Scale bar in A represents 100 μm.

By contrast, treatment with the RAR alpha antagonist (10^{-6} M) turned RA signaling off in the whole explant and had profound effects on lung morphogenesis. Figs. 5C, G show markedly decreased *RARElac*Z signals already at 18 h, and no staining after 72 h treatment. Alpha antagonist-treated explants displayed an irregularly shaped foregut tube. At the prospective lung region no buds or sometimes a truncated bud could be identified; a posterior dilation suggested that the stomach did form (Figs. 5K, 6G). Expression of *Nkx2.1* in the lung field was greatly diminished and no *SftpC* signals were observed in the presumptive lung endoderm (Figs. 6A, C and 7A, B). Consistent with the altered morphogenesis of the foregut tube in the lung field, overall expression of *Fgf10* was decreased and diffuse (Figs. 7G, H). The preserved morphology of the pancreas and thyroid buds and maintained expression of *Pdx1* (Figs. 7D, E) and *Nkx2.1* (Figs. 4A, C) mRNAs in each respective organ primordia, indicated that the developing lung was particularly sensitive to disruption of RAR alpha signaling.

RAR beta agonist maintains lung bud formation in RAR alpha antagonist-treated explants

The absence of *RARElac*Z signals in alpha antagonist-treated cultures at 72 h suggested that endogenous RAR beta signaling depended on RAR alpha activation. We found that *RARElac*Z activity in alpha antagonist-treated (10^{-6} M) explants could be at least transiently maintained by addition of beta agonist (10^{-7} M). At 18 h *RARElac*Z expression was clearly stronger than in explants treated with alpha antagonist alone (Figs. 5C, D). However, by 72 h, activity was no longer detected in any of the explants treated with alpha antagonist, in spite of the presence of beta agonist (Figs. 5G, H). We ascribed this effect to the downregulation of the RAR beta gene consequent to the antagonization of RAR alpha signaling, which was observed after the initial 24 h in culture (data not shown).

Surprisingly, although transient, the activation of beta signaling in cultures treated with alpha antagonist plus beta

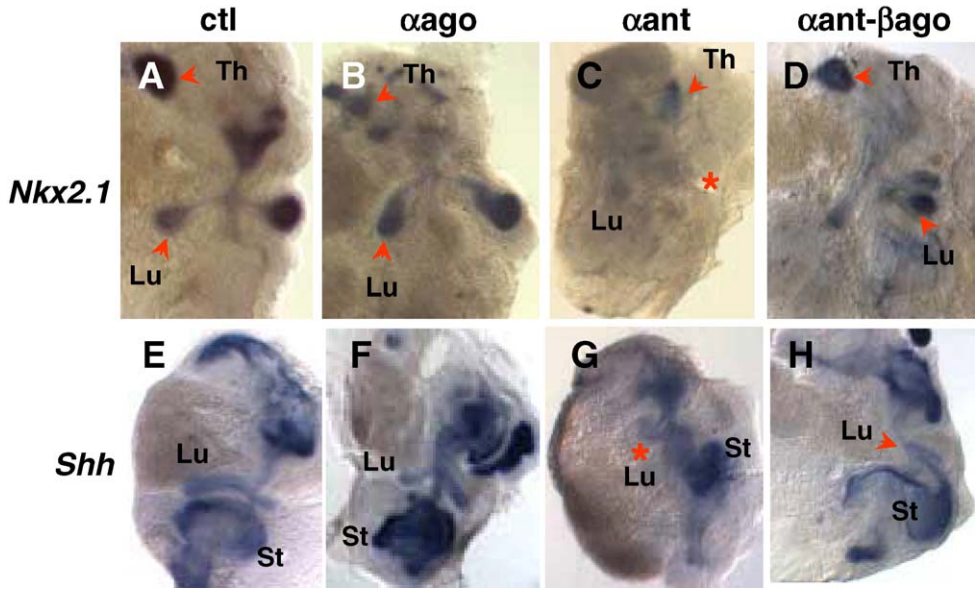

Fig. 6. Whole mount in situ hybridization (WMISH) of *Nkx2.1* (A–D) shows expression unaffected by RAR alpha agonist, but disrupted in the lung field by alpha antagonist. RAR beta agonist rescues the alpha antagonist defect. (E–H) By WMISH *Shh* mRNA is expressed throughout the foregut endoderm of the explant and is not significantly altered by these retinoids.

agonist was sufficient to allow bud formation (Figs. 5L, 6H) and to maintain a program of differentiation characterized by *Nkx2.1* and *SftpC* expression in the lung field (Figs. 6D and 7C). Although not as locally strong as in controls, *Fgf10* signals could be identified in association with lung buds (Figs. 7G, I).

Together, our results suggest that, normally, *Fgf10* expression in the mid foregut is induced by RAR beta-dependent RA signaling, and that Fgf10 is a critical signal for further development of lung progenitor cells. It also confirms previous observations that, once initiated, expression of *Fgf10* becomes independent of RA signaling (Desai et al., 2004). Our data indicated that the beta agonist was effective during an early developmental window to initiate this process.

Excess RAR beta activation induces multiple foci of Fgf10 expression and multiple buds in the lung field

To examine the effects of RAR beta overactivation in lung morphogenesis, we treated foregut explants of the reporter mouse with RAR beta agonist alone (10^{-7} M). Xgal staining of these cultures showed initially (18 h) a broad activation of RA signaling; signals became progressively more restricted to the cardiac mesenchyme and foregut endoderm (Figs. 8A–B). Interestingly, beta agonist stimulation, in the context of normal endogenous, RA signaling resulted in formation of multiple buds in the lung region, without apparently affecting the morphology of the explant elsewhere (Figs. 8C, F, G). *Nkx2.1* analysis confirmed the lung identity of these buds (Fig. 8D). As expected, beta agonist induced multiple ectopic sites of *Fgf10* expression in the mesoderm within the lung field (Figs. 8F, G). This pattern correlated with the appearance of multiple lung buds. Histological sections of *RARElacZ* explants revealed localized signals in the mesoderm associated with the nascent lung buds, where presumably local *Fgf10* is induced (Fig. 8E). In some cases a major, broad domain of *Fgf10* expression seemed to have led to ectopic budding. In other cases, local *Fgf10* signals appeared to be so strong that a bud was ectopically induced from the stomach (Fig. 8G, also H&E in 8H).

Discussion

Early roles of RA in the developing foregut

Here we investigated the initial stages of lung development in a genetic mouse model of RA deficiency. Studies in several species indicate that RA signaling is established in the gastrulating embryo and is required for the initial stages of organogenesis (Rossant et al., 1991; Niederreither et al., 1999; Zile, 2001). RA is a survival factor for the foregut endoderm of quail and chick embryos (Zile, 2001; Ghatpande et al., 2000). In zebrafish, RA signaling controls endodermal specification along the anterior–posterior axis of the foregut, being required for pancreatic and liver cell fates. Treatment of late gastrulation zebrafish embryos with *all-trans* RA results in anterior expansion of the pancreas and liver fields. Conversely, treatment with the pan-RAR antagonist BMS493 blocks the appearance of these domains (Stafford and Prince, 2002). Our current analysis of *Raldh2*$^{-/-}$ mutants provides novel genetic evidence that in mice thyroid and lung can be specified in the absence of RA signaling. We show that early markers of these derivatives can be detected in the foregut of these mutants both in vivo and in vitro. We also confirm recent reports that specification of the ventral pancreas is RA independent (Molotkov et al., 2005; Martin et al., 2005). The different requirement of RA in the developing murine and zebrafish

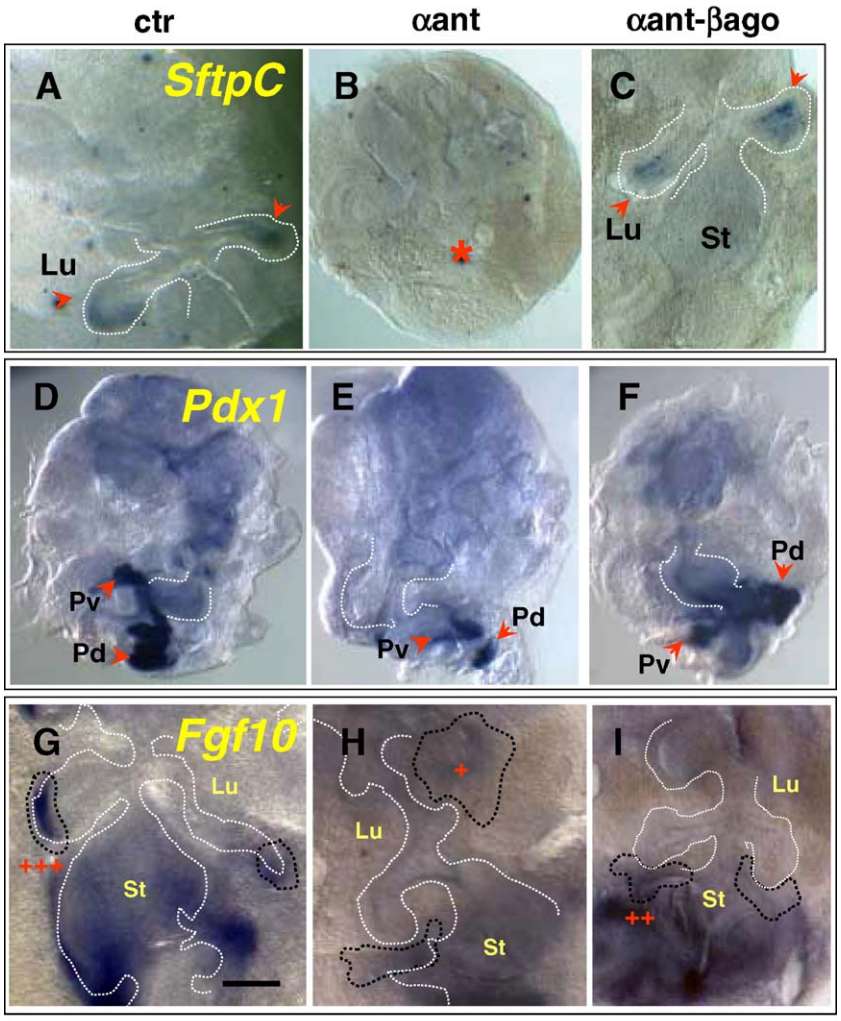

Fig. 7. Differential effects of RAR alpha antagonist in *SftpC* (A, B), and *Pdx1* (D, E) expression (whole mount in situ hybridization, WMISH) under normal endogenous RA signaling. Alpha antagonist prevents *SftpC mRNA* from being induced in the foregut, but does not seem to interfere with pancreatic bud induction and *Pdx1* expression. (C) When combined to RAR alpha antagonist, RAR beta agonist allows formation of *SftpC*-expressing buds, and *Pdx1* expression is unaffected (F). (G–I) *Fgf10* WMISH: strong local expression is depicted in controls (G); alpha antagonist (H) downregulates *Fgf10* and signals become less localized, particularly in the lung region (compare G and H). (I) By adding beta agonist, buds can be seen in the lung field and *Fgf10* expression seems partially restored locally. Number of (+) represents relative intensity of *Fgf10*. Scale bar in panel G represents 150 μm.

models presumably reflects a loss of the RA role in anterior–posterior specification of the foregut, or the appearance of other signaling pathways controlling endodermal specification, as species evolved.

RAR alpha and beta, although interdependent, regulate different events in lung morphogenesis

We have recently reported major differences between RAR alpha and beta effects in the lung when these receptors are constitutively activated in the distal epithelium throughout late gestation in vivo. The study revealed that downregulation of RAR alpha signaling is critical to allow completion of the late program of differentiation that ultimately forms alveolar type I and II cells (Wongtrakool et al., 2003). This work, however, did not focus on the role of these receptors in the early lung. Furthermore, activation of RA signaling was maintained solely in the lung epithelium.

Here, we present novel evidence that during initiation of lung morphogenesis, RAR alpha and beta have distinct functions. By treating *Raldh2*$^{-/-}$ explants with selective RAR agonists we minimized the possibility of functional compensation likely observed in RAR single knock out mice. We show that in *Raldh2*$^{-/-}$ foregut activation of RAR beta, but not RAR alpha, is able to induce *Fgf10* expression in the lung field and trigger bud morphogenesis. The absence of *SftpC* mRNA in alpha agonist-treated explants even after PCR amplification is strongly suggestive of true qualitative differences in the pathways activated by these receptors.

Moreover, our analysis of wild type mice shows that balanced activity of RAR alpha and beta is required for normal lung gene expression and morphogenesis. These experiments provided evidence that shifting this balance towards beta signaling favors *Fgf10* expression and bud formation. This conclusion was supported by the rescue of gene expression and budding in beta agonist-treated *Raldh2*$^{-/-}$ explants, and by the

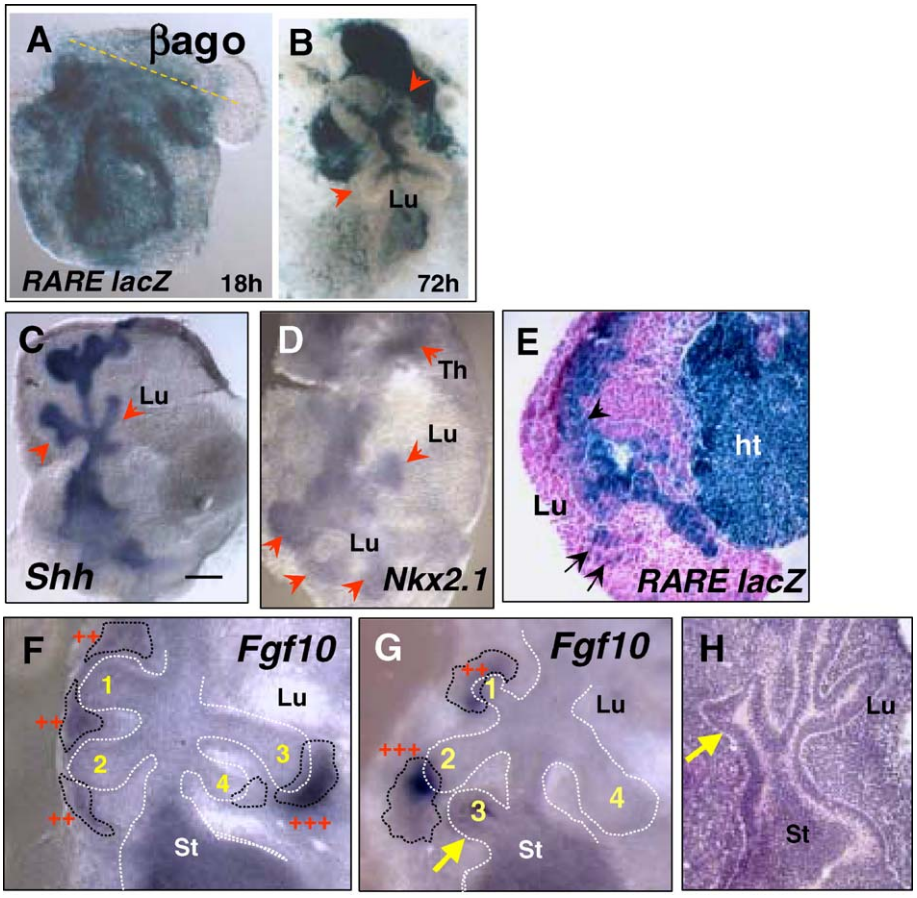

Fig. 8. Effect of RAR beta agonist in RA sufficient foregut explants. (A–B) X-gal staining of *RARElac*Z shows signals broadly induced by beta agonist in the 18 h cultured foregut (left), which becomes more localized to the foregut endoderm at 72 h (B, arrowheads). (C) *Shh* staining by whole mount in situ hybridization (WMISH) reveals multiple lung buds, which express *Nkx2.1* (D). (E) Histological section of beta agonist-treated, X-gal stained *RARElac*Z explant shows staining in foregut endoderm, but also local signals in the mesoderm associated with the nascent lung bud (potential sites of *Fgf10*expression). (F, G) WMISH of *Fgf10*: beta agonist treatment induces multiple ectopic sites of *Fgf10* expression (+ represents signal intensity) and bud formation (numbered) within the lung field. In panel G a bud (#3) is ectopically induced from the stomach (yellow arrow), presumably due to the high local levels of Fgf10. (H) H&E staining of beta agonist-treated explant depicting ectopic bud from the stomach (st). Scale bar in panel C represents 150 μm.

beta agonist-mediated induction of multiple buds in the lung field of wild type foreguts.

The other important observation was that, although acting in different events, RAR alpha and beta are functionally interdependent. Analysis of wild type foreguts showed that if RAR alpha is antagonized, endogenous beta signaling is not activated and *Fgf10* expression is disrupted. The effect could be overcome by providing exogenous beta agonist during the proper developmental window. The unique phenotype obtained with beta agonist treatment (multiple buds), not reproduced by simultaneous activation of all RA receptors by *all trans* RA (Desai et al., 2004), provides additional support for the specificity of the effects described here.

Concluding remarks

There is evidence that lung and liver specification depend on secreted factors from the cardiac mesoderm (Serls et al., 2005; Rossi et al., 2001). Although heart development was greatly disturbed in the *Raldh2*$^{-/-}$mutants, we found that the early liver and ventral pancreatic primordia appeared preserved. Thus, it is likely that putative cardiac factors such as Fgfs other than Fgf10, and bone morphogenetic proteins (Bmps), which at an early stage specify these derivatives, are properly secreted by the heart in mutants. The present study on *Raldh2*$^{-/-}$mutants provides additional support to the idea that, under retinoid deficiency, lung morphogenesis is arrested by the inability of the foregut to selectively induce mesodermal *Fgf10* in the prospective lung field (Desai et al., 2004). Here we show that in these mutants the lung defect can be overcome solely by providing exogenous FGF10, and that this factor is able to maintain an early program of lung endodermal differentiation characterized by *Nkx2.1* and *SftpC* expression in lung progenitor cells.

By activating selective RARs with retinoid agonists in *Raldh2*$^{-/-}$mutants, we found that it is possible to gain insights into individual functions of these receptors that the RAR knockout approach was not able to provide. Together, the data favor the hypothesis that RAR alpha signaling serves to fine tune the RAR beta effects. Accordingly, the RAR alpha role would include maintaining adequate levels of RAR beta expression and limiting activation of RAR beta and its targets

to their proper domains. Studies are underway to further investigate pathways differentially regulated by these RARs in the early lung.

Acknowledgments

We are indebted to Janet Rossant for the *RARElacZ* mouse line and Chris Zusi (Bristol-Myers Squibb) for providing us with the synthetic retinoids, Parviz Minoo, Nobuyuki Itoh, Andrew McMahon, and Chris Wright for cDNA clones. We thank Molly Weaver, Ross Metzger, Hérnan Espinoza for helpful discussions. We also thank Chun Li and Xiuzhi Tang for excellent technical assistance. This work was supported by grants from NIH/NHLBI (1RO1HL/HD67129-01 and 2PO1HL47049: W.V.C), and by a GlaxoSmithKline Pulmonary Fellowship Award (T.D.).

References

Bhushan, A., Itoh, N., Kato, S., Thiery, J.P., Czernichow, P., Bellusci, S., Scharfmann, R., 2001. Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128, 5109–5117.

Cardoso, W.V., 2001. Molecular regulation of lung development. Annu. Rev. Physiol. 63, 471–494.

Cardoso, W.V., Itoh, A., Nogawa, H., Mason, I., Brody, J.S., 1997. FGF-1 and FGF-7 induce distinct patterns of growth and differentiation in embryonic lung epithelium. Dev. Dyn. 208, 398–405.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. 10, 940–954.

Chapellier, B., Mark, M., Messaddeq, N., Calleja, C., Warot, X., Brocard, J., Gerard, C., Li, M., Metzger, D., Ghyselinck, N.B., Chambon, P., 2002. Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled. EMBO J. 21, 3402–3413.

Chazaud, C., Dolle, P., Rossant, J., Mollard, R., 2003. Retinoic acid signaling regulates murine bronchial tubule formation. Mech. Dev. 120, 691–700.

Clagett-Dame, M., DeLuca, H.F., 2002. The role of vitamin A in mammalian reproduction and embryonic development. Annu. Rev. Nutr. 22, 347–381.

De Moerlooze, L., Spencer-Dene, B., Revest, J., Hajihosseini, M., Rosewell, I., Dickson, C., 2000. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal–epithelial signalling during mouse organogenesis. Development 127, 483–492.

Desai, T.J., Malpel, S., Flentke, G.R., Smith, S.M., Cardoso, W.V., 2004. Retinoic acid selectively regulates Fgf10 expression and maintains cell identity in the prospective lung field of the developing foregut. Dev. Biol. 273, 402–415.

Dickman, E.D., Thaller, C., Smith, S.M., 1997. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. Development 124, 3111–3121.

Dilworth, F.J., Fromental-Ramain, C., Remboutsika, E., Benecke, A., Chambon, P., 1999. Ligand-dependent activation of transcription in vitro by retinoic acid receptor alpha/retinoid X receptor alpha heterodimers that mimics transactivation by retinoids in vivo. Proc. Natl. Acad. Sci. U. S. A. 96, 1995–2000.

Dolle, P., Ruberte, E., Leroy, P., Chambon, P., 1990. Retinoic acid receptors and cellular retinoid binding proteins: I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development 110, 1133–1151.

Gehin, M., Vivat, V., Wurtz, J.M., Losson, R., Chambon, P., Moras, D., Gronemeyer, H., 1999. Structural basis for engineering of retinoic acid receptor isotype-selective agonists and antagonists. Chem. Biol. 6, 519–529.

Ghatpande, S., Ghatpande, A., Zile, M., Evans, T., 2000. Anterior endoderm is sufficient to rescue foregut apoptosis and heart tube morphogenesis in an embryo lacking retinoic acid. Dev. Biol. 219, 59–70.

Harada, H., Toyono, T., Toyoshima, K., Yamasaki, M., Itoh, N., Kato, S., Sekine, K., Ohuchi, H., 2002. FGF10 maintains stem cell compartment in developing mouse incisors. Development 129, 1533–1541.

Hogan, B.L., 1999. Morphogenesis. Cell 96, 225–233.

Kastner, P., Messaddeq, N., Mark, M., Wendling, O., Grondona, J.M., Ward, S., Ghyselinck, N., Chambon, P., 1997. Vitamin A deficiency and mutations of RXRalpha, RXRbeta and RARalpha lead to early differentiation of embryonic ventricular cardiomyocytes. Development 124, 4749–4758.

Kimura, S., Ward, J.M., Minoo, P., 1999. Thyroid-specific enhancer-binding protein/thyroid transcription factor 1 is not required for the initial specification of the thyroid and lung primordia. Biochimie 81, 321–327.

Li, E., Sucov, H.M., Lee, K.F., Evans, R.M., Jaenisch, R., 1993. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. Proc. Natl. Acad. Sci. U. S. A. 90, 1590–1594.

Malpel, S., Mendelsohn, C., Cardoso, W.V., 2000. Regulation of retinoic acid signaling during lung morphogenesis. Development 127, 3057–3067.

Martin, P.J., Lardeux, V., Lefebvre, P., 2005. The proliferating cell nuclear antigen regulates retinoic acid receptor transcriptional activity through direct protein–protein interaction. Nucleic Acids Res. 33, 4311–4321.

Matt, N., Ghyselinck, N.B., Wendling, O., Chambon, P., Mark, M., 2003. Retinoic acid-induced developmental defects are mediated by RARbeta/RXR heterodimers in the pharyngeal endoderm. Development 130, 2083–2093.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., Mark, M., 1994a. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 120, 2749–2771.

Mendelsohn, C., Mark, M., Dolle, P., Dierich, A., Gaub, M.P., Krust, A., Lampron, C., Chambon, P., 1994b. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. Dev. Biol. 166, 246–258.

Mic, F.A., Haselbeck, R.J., Cuenca, A.E., Duester, G., 2002. Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of Raldh2 null mutant mice. Development 129, 2271–2282.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila* branchless. Genes Dev. 12, 3156–3161.

Minoo, P., Su, G., Drum, H., Bringas, P., Kimura, S., 1999. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(−/−) mouse embryos. Dev. Biol. 209, 60–71.

Mollard, R., Ghyselinck, N.B., Wendling, O., Chambon, P., Mark, M., 2000. Stage-dependent responses of the developing lung to retinoic acid signaling. Int. J. Dev. Biol. 44, 457–462.

Molotkov, A., Molotkova, N., Duester, G., 2005. Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev. Dyn. 232, 950–957.

Niederreither, K., Subbarayan, V., Dolle, P., Chambon, P., 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat. Genet. 21, 444–448.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P., Dolle, P., 2000. Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development 127, 75–85.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., Dolle, P., 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 128, 1019–1031.

Perl, A.K., Tichelaar, J.W., Whitsett, A.J., 2002. Conditional gene expression in the respiratory epithelium of the mouse. Transgenic Res. 11, 21–29.

Rossant, J., Zirngibl, R., Cado, D., Shago, M., Giguere, V., 1991. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev. 5, 1333–1344.

Rossi, J.M., Dunn, N.R., Hogan, B.L., Zaret, S.K., 2001. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev. 15, 1998–2009.

Ruberte, E., Dolle, P., Chambon, P., Morriss-Kay, G., 1991. Retinoic acid

receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development 111, 45–60.

Schoenermark, M.P., Mitchell, T.I., Rutter, J.L., Reczek, P.R., Brinckerhoff, E.C., 1999. Retinoid-mediated suppression of tumor invasion and matrix metalloproteinase synthesis. Ann. N. Y. Acad. Sci. 878, 466–486.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N., Kato, S., 1999. Fgf10 is essential for limb and lung formation. Nat. Genet. 21, 138–141.

Serls, A.E., Doherty, S., Parvatiyar, P., Wells, J.M., Deutsch, G.H., 2005. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development 132, 35–47.

Shannon, J.M., Hyatt, B.A., 2004. Epithelial–mesenchymal interactions in the developing lung. Annu. Rev. Physiol. 66, 625–645.

Stafford, D., Prince, V.E., 2002. Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr. Biol. 12, 1215–1220.

Vivat-Hannah, V., You, D., Rizzo, C., Daris, J.P., Lapointe, P., Zusi, F.C., Marinier, A., Lorenzi, M.V., Gottardis, M.M., 2001. Synergistic cytotoxicity exhibited by combination treatment of selective retinoid ligands with taxol (Paclitaxel). Cancer Res. 61, 8703–8711.

Wert, S.E., Glasser, S.W., Korfhagen, T.R., Whitsett, J.A., 1993. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. Dev. Biol. 156, 426–443.

Wilson, J.G., Roth, C.B., Warkany, J., 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. Am. J. Anat. 92, 189–217.

Wongtrakool, C., Malpel, S., Gorenstein, J., Sedita, J., Ramirez, M.I., Underhill, T.M., Cardoso, W.V., 2003. Down-regulation of retinoic acid receptor alpha signaling is required for sacculation and type I cell formation in the developing lung. J. Biol. Chem. 278, 46911–46918.

Zile, M.H., 2001. Function of vitamin A in vertebrate embryonic development. J. Nutr. 131, 705–708.